PATENT

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Applicants	Achilefu et al.
Title	**RECEPTOR AVID EXOGENOUS OPTICAL AND THERAPEUTIC AGENTS**
Group Art Unit:	1618
Examiner	Jones, Dameron Levest
Attorney Docket No.	1448.2 H US (073979.40)

December 22, 2009

DECLARATION OF RAGHAVAN RAJAGOPALAN, Ph.D.
PURSUANT TO 37 C.F.R. §1.132

I, Raghavan Rajagopalan, declare as follows:

1. I am an inventor in the above-identified patent application, an employee of the Assignee Mallinckrodt Inc., and am also a registered Patent Agent.

2. I hold a Ph.D. in Organic Chemistry from Columbia University. I have 25 years of experience in the synthesis and use of compounds for medical diagnosis and therapy, which is the subject of the application.

3. I have read the September 23, 2009 Office Action and applied references, and understand the Examiner's position.

4. I am an inventor on, and Mallinckrodt Inc. is the Assignee of, U.S. Patent No. 6,423,547, which the Examiner applies to reject claims 32-35 under 35 U.S.C. 103(a). The Assignment is recorded in the U.S. Patent and Trademark Office at reel/frame 011166/0819.

5. Mallinckrodt Inc. is the Assignee of the present application. The Assignment is recorded in the U.S. Patent and Trademark Office at reel/frame 015174/0425.

6. Because the present invention and the invention disclosed but not claimed in the '547 patent was derived by me, and thus is not any invention "by another", the rejection under 35 U.S.C. §103(a) is overcome.

7. Regarding the rejection of claims 32-35 under 35 U.S.C. §112 ¶1 as not described, I respectfully disagree. The Examiner states

> In addition, according to Licha et al (US Patent No. 6,083,485), the classes of cyanine dyes are structurally different. For example, in column 5, line 1, Licha et al disclose a general formula for a cyanine dye. In columns 6-7, bridging paragraph, Licha disclose a general formula for merocyanine dyes. It should be noted that the structure of the merocyanine dyes are different from those having the general cyanine dye formula. Other structurally different cyanine dyes include those found in columns 10-11, bridging paragraph (Licha et al) that differ in how the variable Q is defined.

8. Licha describes the col. 5 line 1 structure, not as "a general formula of a cyanine dye", but rather as "a cyanine dye of the general formula IIa". Thus Licha's col. 5 line 1 structure is one of a general formula, in accord with the Hawley Chemical Dictionary definition of a cyanine dye.

9. The nomenclature of cyanine dyes is known to a person of ordinary skill in the art. In support, I have attached Ernst et al., Cytometry 10 (1989) 3, and point the Examiner to the Abstract, p. 3 col. 2 bridging p. 4 col. 1, and FIG. 1. The term "cyanine" is a root name referring to all molecules that have a charged nitrogen on the left side of the molecule, connected to a polymethine group.

10. When the right side of the polymethine group is connected to a nitrogen, the molecule retains the unmodified name cyanine. Thus, a cyanine can be written as, e.g., $RR'N^{+}=C-(C=C)-C=C-NRR"$.

11. When the right side of the polymethine group is connected to other than a nitrogen, by convention, a prefix is added to the term "cyanine" to indicate the type of modification. For example, merocyanine is a cyanine because it contains the core cyanine structure (a charged nitrogen on the left side of the molecule, connected to a polymethine group), with the right side of the polymethine group connected to an oxygen. Thus, a merocyanine can be written as, e.g., $RR'N+=C-(C=C)-C=C)-0^{-}$.

12. Cyanine dyes are resonance structures; the positive charge moves to the other compound connected to the polymethine group. The identity of the R groups is irrelevant as long as the methine groups allows the charge to move.

13. The term isocyanine is, by convention, a specific name used with a specific compound related to quinoline blue. Quinoline blue is a compound having two quinolines connected 4, 4' by a methine group. The prefix "iso" on the root "cyanine" indicates that the quinoline rings are attached differently (2, 4') to the methine.

14. Thus, in my opinion, claims 32-35 are sufficiently described.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the subject application or any patent issued thereon.

December 22, 2009
Date

Raghavan Rajagopalan
Raghavan Rajagopalan, Ph.D.

753601

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Patent Assignment Abstract of Title

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Total Assignments: 1

Patent #: 6423547 **Issue Dt:** 07/23/2002 **Application #:** 09646765 **Filing Dt:** 09/21/2000

Inventors: Raghavan Rajagopalan, Joseph E. Bugaj, Richard Bradley Dorshow, Samuel I. Achilefu

Title: NON-COVALENT BIOCONJUGATES USEFUL FOR DIAGNOSIS AND THERAPY

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Conveyance: ASSIGNMENT OF ASSIGNORS INTEREST (SEE DOCUMENT FOR DETAILS).

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DIANE RUSSELE, PARALEGAL
ASSIGNMENT DIVISION
OFFICE OF PUBLIC RECORDS

Cytometry 10:3–10 (1989)

Cyanine Dye Labeling Reagents for Sulfhydryl Groups[1]

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Cyanine and merocyanine dyes are introduced as new fluorescent reagents for covalently labeling proteins and other biomolecules. These dyes, which contain iodoacetamide functional groups, have high extinction coefficients and moderate quantum yields. A major advantage of these polymethine dyes is the easy manipulation of their spectral properties during synthesis. Cyanines containing reactive functional groups can be made with absorption maxima ranging from < 500 nm to > 750 nm. This property opens additional regions of the spectrum for experiments involving the simultaneous multicolor analysis of different fluorescent probes. The cyanines, which are relatively insensitive to solvent property changes, are complemented by the merocyanines, which are keen indicators of solvent polarity.

Key terms: **Fluorescence, covalent labeling reagents, merocyanine dyes, fluorescent analog cytochemistry, red and infrared fluorescent probes**

Fluorescence techniques have been used in a wide range of biological applications. The primary advantages of these methods are their high sensitivity and specificity and the ability to monitor events with high temporal resolution. When combined with microscopy, fluorescence methods are also capable of high spatial resolution. Since the detection of fluorescence is noninvasive, dynamic processes can be followed in living cells and organisms.

Fluorescent analog cytochemistry is a method developed to monitor the activity and distribution of specific cellular components in living cells (25). This technique involves covalently coupling a fluorescent probe to a purified native cellular component, such as a protein. The analog is characterized in vitro, incorporated into cells and then characterized in vivo. Many fluorescent analogs have already been prepared, including analogs of actin (26), tubulin (17), calmodulin (30), and phospholipids (21).

The variety and uses of fluorescent reagents capable of covalently labeling biomolecules have recently been reviewed (15). Waggoner discussed the ideal properties of fluorescent probes giving the maximum detectability of the probe with minimal perturbation of the function of the target molecule (28). Most of the reagents now available have absorption maxima at wavelengths less than 500 nm. The autofluorescence from normal cellular constituents is also highest in this spectral region (2). The amount of autofluorescence of a typical cultured fibroblast cell is equivalent to 35,000 molecules of fluorescein (19). Therefore, the detection of a fluoresceinated analog in these cells can be limited by the background 'noise.' It is also difficult to monitor simultaneously several different fluorescent parameters using the restricted selection of labeling reagents currently available (8).

The cyanine and merocyanine polymethine dyes have many of the properties presented as ideal for fluorescent probes. They have high extinction coefficients (> 100,000/mol cm) and moderate quantum yields and photostability. These dyes can be synthesized with excitation maxima ranging from about 500 nm to beyond 750 nm. Cyanine dyes (I, Fig. 1), are composed of two quaternized heteroaromatic bases (A and A') joined by a polymethine chain. These compounds have a cationic character due to the delocalized positive charge of the chromophore. The spectral properties of cyanine dyes are only slightly sensitive to solvent changes. Merocyanine dyes (II, Fig. 1), have a polymethine chain linking a quaternized basic nucleus (A) and a ketomethylene-derived nucleus (B). As demonstrated, merocyanines

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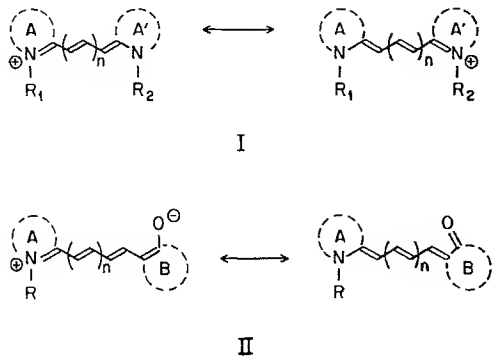


FIG. 1. Generalized structures of cyanine (I) and merocyanine (II) dyes. Notice that the cyanines contain a delocalized positive charge while the merocyanines can exist in both nonpolar and dipolar forms.

have both non-polar and zwitterionic resonance forms. The spectral properties of merocyanines are very sensitive to changes in solvent polarity. The syntheses and properties of these polymethine dyes have been well documented (10, 14). These dyes have previously been used to monitor membrane potentials (27) and to label lipid bilayers (20) and low density lipoprotein (3).

This is the first of a series of reports specifying the syntheses of cyanine and merocyanine dyes which are covalent labeling reagents for biological molecules. Our initial account describes cyanines and merocyanines containing iodoacetamido (IA) and iodoacetamidomethyl ($IACH_2$) groups which react rapidly and specifically with sulfhydryl groups at neutral pH. Although -SH groups are rare in extracellular proteins, these groups are common in proteins from the reducing environment inside cells. These new reactive polymethine dyes are therefore well-suited as fluorescent tagging reagents for fluorescent analog cytochemistry studies. The high selectivity of the iodoacetyl group for sulfhydryls allows easy handling of these dyes without loss of activity. Use of these reagents opens additional spectral windows allowing several analogs to be monitored with minimal interference by autofluorescence or resonance energy transfer.

MATERIALS AND METHODS

Spectroscopic Measurements

Absorption spectra were recorded on either a Perkin-Elmer Model 575 or a Varian Model 2200 spectrophotometer. Some fluorescence measurements were performed on a Hitachi Perkin-Elmer MPF-3 spectrofluorimeter equipped with either a Hamamatsu R-446 or R-928 photomultiplier tube. Other fluorescence measurements were obtained with a Spex Fluorolog-2 system. Infrared spectra were collected on a Nicolet Model 5DXB FT-IR spectrometer. Proton NMR spectra were taken on a Bruker 300 FT-NMR spectrometer.

Fluorescence Quantum Yield Determinations

The determinations of emission maxima and fluorescence intensities were carried out with dilute dye solutions exciting on a short-wavelength shoulder of the main absorption band. The absorbance of the sample was always less than 0.05. The fluorescence intensities were corrected for the intensity of the exciting light and the sensitivity of the detection system. The quantum yields were calculated relative to standard solutions in methanol of rhodamine 6G with $\phi = 0.95$ (9) and a cyanine dye, 3,3′-diethyl-thiadicarbocyanine with $\phi = 0.33$ (22). All sample dye solutions were made in 95% ethanol by diluting methanolic stock solutions.

Thin Layer Chromatography

Analytical thin layer chromatography of reactive dyes and their conjugates was performed on silica gel sheets, developed with ethanol-acetic acid (19:1), and on C18-reverse phase TLC plates developed with methanol-water (2:1).

Conjugation of Reactive Dyes

To a selected dye (~ 30 mg) dissolved in ethanol (6 ml) was added 3 ml aqueous cysteine (0.1M, pH 8). The reaction mixture was incubated for 20 min at room temperature, then concentrated by rotary evaporation to remove the alcohol. The resulting aqueous mixture was passed through a C18-Sep-Pak cartridge (Waters Associates, Milford, MA). After washing the cartridge with water (3 × 5 ml), the dye-cysteine conjugate was eluted with methanol. Evaporation of the methanol yielded almost pure conjugate (~ 25 mg).

Reaction of CY5.4-IA With F-Actin

The labeling of polymerized actin was achieved by a modification of the method of Wang and Taylor (29). Monomeric G-actin was dialyzed vs. ascorbate buffer to remove excess DTT, and then polymerized with high salt and magnesium ions. A 10-fold molar excess of dye (adsorbed at 10% w/w on celite) was added to the actin preparation. The mixture was stirred magnetically for 5 min and rocked gently for 30 min. After removal of the celite by a low speed spin, the labeling reaction was quenched by the addition of 2 mM cysteine. After a 15 min incubation the F-actin was sedimented by high speed centrifugation. The resulting dark blue pellet was resuspended in low salt buffer and depolymerized with potassium iodide. The actin (G-form) was purified by gel permeation chromatography over a G-25 Sephadex column, repolymerized and sedimented by high speed centrifugation. The recovered actin was depolymerized with low ionic strength Tris buffer and then dialyzed vs. Pipes injection buffer. The dye/protein ratio of the sample was measured, the sample was aliquoted, and then stored in liquid nitrogen until needed. An aliquot of this actin was assayed for polymerization ability following freezing. The biological activity of the cyanine-labeled

actin was tested by monitoring the incorporation of the protein into stress fibers after microinjection into swiss albino 3T3 cells (1). For observation of the cyanine labeled cells, the microscope was fitted with an excitation filter having a 30 nm bandpass centered at 620 nm and with an emission barrier filter having a 50 nm bandpass centered at 675 nm.

Preparation of Reactive Dyes

Procedures for the synthesis of representative dyes are given below. Overall yields for the multistep syntheses ranged from about 8% to 20% for the cyanine dyes and from 25% to 50% for the merocyanine dyes. Formation of the appropriate chromophore for each haloacetamido dye was indicated by its characteristic absorption maximum (5–7,24). The purified products had molar absorption coefficients and emission maxima comparable to unsubstituted model dyes synthesized in this laboratory. IR spectra of the haloacetyl-cyanines confirmed the formation of the cyanine chromophores. However the strong IR bands of the chromophores in most cases masked the transitions of the other functional groups. Even the amide I carbonyl stretching vibrations gave relatively weak bands. These unsymmetrical cyanines gave complex NMR spectra containing poorly resolved peaks which proved impractical for the characterization of the products. Although the reactive dyes showed only single components by thin layer chromatography, elemental analyses gave inconsistent results due to the presence of variable amounts of inorganic salts. These salts did not interfere with the reactivity of the dyes which was demonstrated by the conjugation reactions described above.

CY5.1-IA (V). 2,3,3-Trimethyl-(3H)-indole (1.6g, 10 mmol) and N-(3-bromopropyl)-phthalimide (2.7g, 10mmol) were heated at 110°C in a sealed tube for 4 h. The resulting red viscous mass, which solidified on cooling, was washed with ether (2 × 20 ml), then dried. The 1-(3-phthalimidopropyl)-2,3,3-trimethyl-indolinium bromide was refluxed for 5 h in 35 ml concentrated hydrochloric acid. The hydrolyzate was allowed to stand at room temperature for 2 d and the crystalline phthalic acid was filtered out. Basification of the filtrate with concentrated ammonium hydroxide caused a yellow precipitate to separate. The solid was collected, washed with water, and dried under vacuum. The dried residue was dissolved in dichloromethane (100 ml) and cooled on ice. Triethylamine (1.94 ml) was added followed by a solution of 0.57 ml chloroacetylchloride in dichloromethane (100 ml) during 1 h. The mixture was allowed to stand at room temperature overnight, washed with water (2 × 75 ml), dried over anhydrous sodium sulfate and concentrated to give 1.7g (~ 4.6mmol) of a light red semi-solid (III).

2,3,3-Trimethyl-(3H)-indole (0.48g, 3.0mmol) and 1,4-butanesultone (0.42g, 3.1mmol) were heated for 3 h in a stoppered flask at 120°C. The viscous red mass was cooled, washed with ether (2 × 5 ml), and dried under vacuum. The product was dissolved in 10 ml acetic anhydride, 0.77g (3.0mmol) of malonaldehyde dianilide hydrochloride (IV) was added (16), and the mixture was heated at 125°C for 30 min. The solution of anhydro 1-(4-sulfobutyl)-2-(4-acetanilino-1,3-butadienyl)-indolinium hydroxide (V) was cooled to room temperature and used without further purification.

The solution of anil (V) was mixed with 0.8g (2.1mmol) of the chloroacetamidoindoline (III). After the mixture was heated to 120°C for 20 min and then cooled on ice, it was diluted with ether (150 ml). The precipitate was collected and chromatographed over silica gel (85g). Elution with 30% ethanol in acetone yielded 0.64g (1.0mmol) of pure chloroacetamido dye, R_f 0.3 (acetone-ethanol 4:1). This dye (0.37g) was refluxed in methanol (80 ml) containing 0.75g (5mmol) sodium iodide for 4 h. The mixture was cooled on ice and then filtered. The filtrate was concentrated to dryness in vacuo. The residue was washed with chloroform (20 ml), acetone (3 × 2 ml), and hexanes (2 × 5 ml) and then dried. Pure CY5.1-IA (VI) was obtained, m.p. 190-193°C. IR (KBr) 925, 1,018, 1,041, 1,103, 1,141, 1,168, 1,338, 1,386, 1,455, 1,492, and 1,654 (amide I band) cm^{-1}.

CY5.6-IA (IX). 5-Chloroacetamidomethyl-1,3,3-trimethyl-2-methylene-indoline (VII) was prepared as described previously (13). 2-Methyl-benzothiazole (1.5g, 10mmol) and propane-1,3-sultone (1.5g, 12mmol) were heated at 120°C for 6 h. The resulting solid mass was triturated with ether, collected, and dried to give almost white crystals (2.55g, 9.4mmol). This material was mixed with 2.6g (10mmol) malonaldehyde dianilide hydrochloride (IV) and acetic anhydride (10 ml), then heated at 120°C for 30 min. The cooled reaction mixture was diluted with 50 ml ether and the ethereal layer decanted. The remaining dark red semi-solid mass was chromatographed over silica gel, eluting with a chloroform-ethanol step gradient. The product was recovered in 20% ethanol, yielding 1.6g (3.6mmol) of pure anhydro 1-(3-sulfopropyl)-2-(4-acetanilino-1,3-butadienyl)-benzothiazolium hydroxide (VIII).

The benzothiazolium anil (VIII) (0.4g, 0.9mmol) and 0.4g (1.4mmol) of the chloroacetamidomethyl-indoline (VII) were dissolved in 15 ml acetic anhydride and stirred for 30 min at room temperature followed by 2 min at 100°C. The resulting dye was precipitated with ether (30 ml) and purified by chromatography over silica gel. Elution with chloroform-ethanol (4:1) yielded pure chloroacetamidomethyl dye (0.1g, 0.2mmol). Refluxing this dye with methanolic sodium iodide as described above gave pure CY5.6-IA (IX). IR (KBr) 937, 1,017, 1,037, 1,106, 1,153, 1,321, 1,370, 1,459, 1,474, 1,508, and 1,656 (amide I band) cm^{-1}.

CY7.4-IA (XI). Butane-1,4-sultone (0.43g, 3.2mmol) and 2,3,3-trimethyl-(3H)-indole (0.5g, 3.1mmol) were heated for 3 h in a stoppered flask at 120°C. The resulting viscous mass was washed with ether (2 × 5 ml) and dried. To this material was added 0.85g (3.0mmol) of glutaconaldehyde dianilide hydrochloride (11) and acetic anhydride (10 ml). The mixture was heated at 120°C for 30 min and then cooled. This solution of anhydro 1-(4-sulfobutyl)-2-(6-acetanilino-1,3,5-hexatrienyl)-indolinium hydroxide (X) was used promptly without purification.

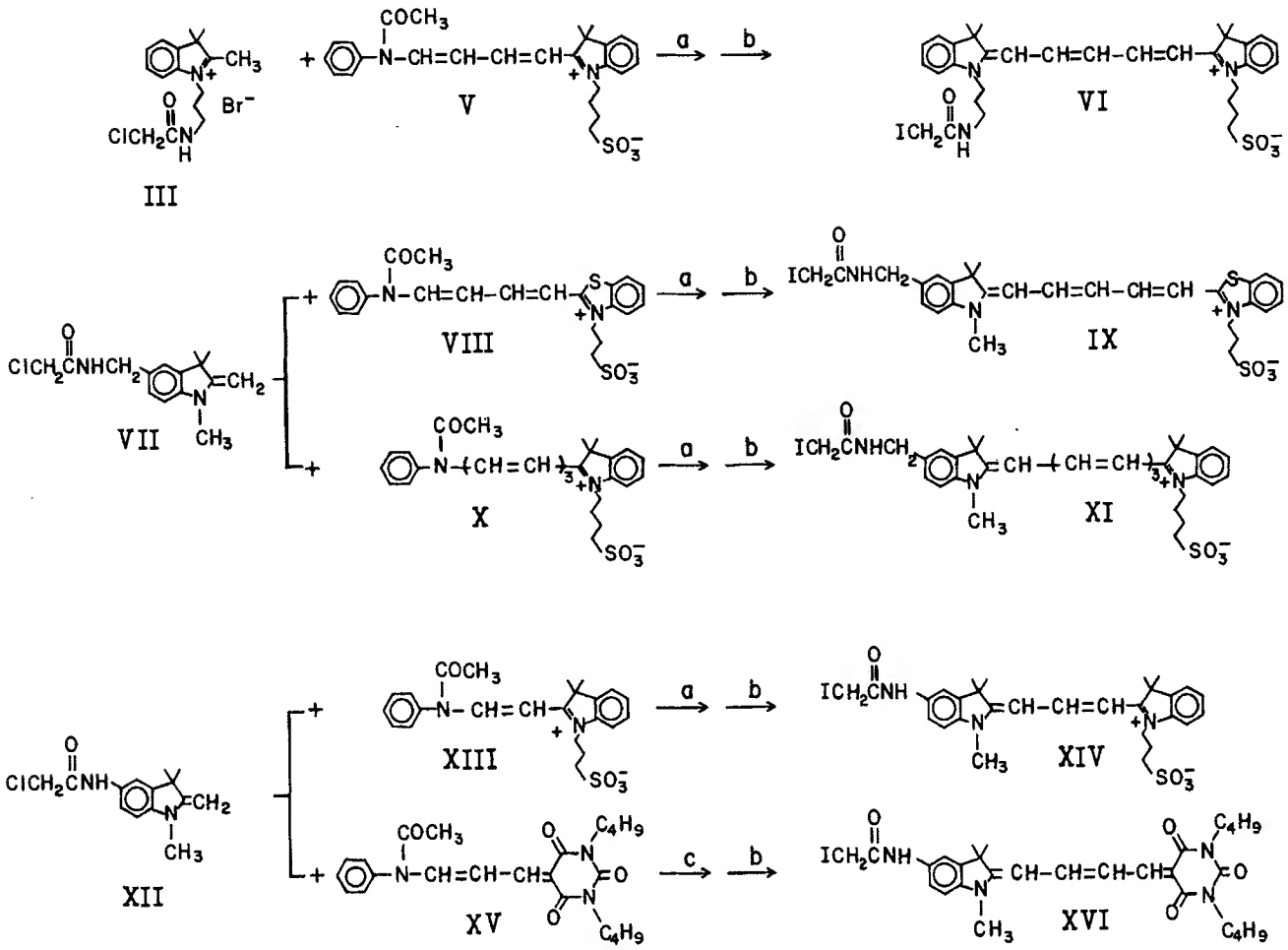


FIG. 3. Synthetic schemes for representative iodoacetamido polymethine dyes. a, acetic anhydride; b, NaI/methanol; c, methanol.

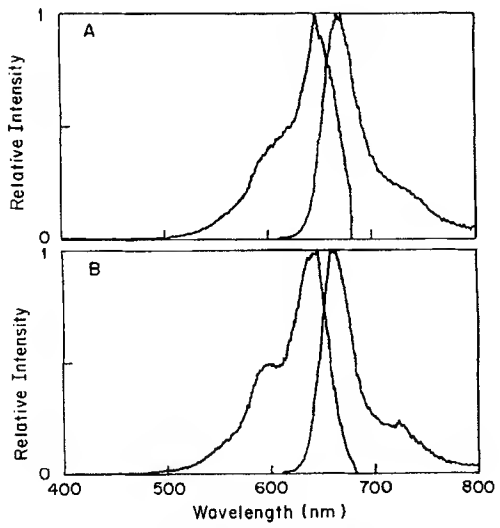


FIG. 4. Excitation (left) and emission (right) spectra for a) CY5.4-G-actin in Buffer E (29) and b) free CY5.4-IA in Buffer E. The excitation spectra were collected by monitoring the emission intensity at 690nm. The emission spectra were obtained by exciting at 600nm. The spectral bandpass of both monochromators was 1 nm.

Polymethine dyes containing different terminal heterocyclic groups (Y = O, S, $C(CH_3)_2$) exhibit different absorption and fluorescence maxima, although the effect is smaller than that observed with the vinylene homologs. The greatest differences are seen between oxygen heterocycles and sulfur heterocycles. The cyanines CY5.5-IA and CY5.6-IA show a 24 nm difference while the merocyanines MC4.1-IA and MC4.2-IA exhibit a 40 nm difference. The dependence of spectra on the terminal heterocycle enables manipulation of the absorption maxima in probes designed for specific applications. The spectral properties of probes can be fine tuned to give maximal overlap for resonance energy transfer experiments or to give minimal overlap between probes in multiple parameter experiments. Other characteristics of these dyes depend on Y. For example, indolinyl cyanines show a greater photostability than other similar cyanines (24). In our experience benzothiazolyl cyanines are more susceptible to oxidative decomposition, both photolytic and chemical, than the benzoxazolyl dyes, and the oxygen containing heterocycles generally have larger quantum yields than the sulfur containing heterocycles.

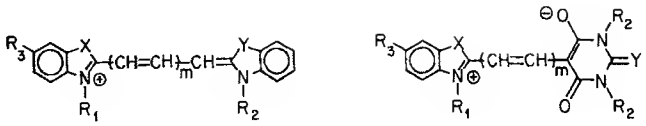
A solution of 1.1g (3.9mmol) chloroacetamidomethyl indoline (VII) in 5 ml acetic anhydride was added to the solution of the anil (X), and the mixture was heated for 30 min at 120°C. The product was precipitated by the addition of ether (70 ml). The oily residue was twice dissolved in ethanol (5 ml) and reprecipitated with ether (30 ml). The resulting oil was chromatographed over silica gel with a chloroform-ethanol step gradient elution. The desired product eluted in 20% ethanol. Concentration of the appropriate fractions gave 0.35g (0.55mmol) of chloroacetamido dye. Treatment of this product (0.32g, 0.50mmol) with sodium iodide (0.19g, 1.3mmol) in boiling methanol as previously described yielded 0.35g (0.48mmol) of CY7.4-IA (XI). IR (KBr) 925, 999, 1,032, 1,085, 1,097, 1,138, 1,412, 1,447, 1,515, 1,541, and 1,656 (amide I band) cm^{-1}.

CY3.2-IA (XIV). 5-Chloroacetylamino-indoline (XII) was prepared by the method of Gale and Wilshire (12.) Propane-1,3-sultone (1.3g, 11mmol) and 2,3,3-trimethyl-(3H)-indole (1.7g, 11mmol) were heated at 100°C for 3 h. The mixture, which solidified on cooling, was washed with ether (2 × 5 ml) and dried in vacuo. To this solid was added 2.0g (10mmol) of N,N′-diphenylformamidine and acetic anhydride (12 ml). The mixture was heated at 120°C for 30 min, cooled, and diluted with ether (30 ml). The solid product was collected and crystallized from ethanol-ether giving 1.5g (3.5mmol) of the anil (XIII), m.p. 308–310°C.

The chloroacetamido indoline (XII) (0.8g, 3.0mmol) and the anil (XIII) (1.3g, 3.1mmol) were dissolved in 15 ml acetic anhydride, heated for 10 min at 120°C, then cooled to room temperature. The reaction mixture was diluted with ether (40 ml). The red solid was collected and dried yielding 1.6g (~ 2.5mmol) of almost pure chloroacetamido dye. This dye (1.4g, ~ 2.2mmol) was refluxed for 2.5 h in methanol (150 ml) containing 1.4g (9.3mmol) sodium iodide. After concentration of the reaction mixture to 10 ml, the product was precipitated by the addition of acetone (200 ml) to give 1.7g of crude product. Chromatography over silica gel with ethanol-chloroform (3:7) elution yielded 0.8g (1.2mmol) of pure CY3.2-IA (XIV), m.p. 245–250°C, R_f 0.37 (acetonitrile-ethanol 2:1). IR (KBr) 929, 1,035, 1,115, 1,152, 1,215, 1,370, 1,415, 1,458, 1,475, 1,560, and 1,680 (amide I band) cm^{-1}.

MC4.1-IA (XVI). 1,3-Dibutyl-barbituric acid was synthesized from 1,3- di-n-butyl urea and diethylmalonate by the method of Brooker et al. (4). 12g (50mmol) of the barbituric acid, 13g (50mmol) of malonaldehyde dianilide hydrochloride (IV), and 4.1g (50mmol) of sodium acetate were refluxed in acetic anhydride (75 ml) for 20 min. The reaction was cooled to 0°C and a mixture of ice and methanol was added until a precipitate began to form. The precipitate was collected, washed with cold methanol, and dried giving 12.8g (~ 31mmol) of brownish yellow anil (XV).

0.53g (2.0mmol) of the chloroacetamido indoline (XII) and 0.83g (2.0mmol) of the barbituric acid anil (XV) were stirred in methanol at room temperature for 20 h. The mixture was concentrated and chromatographed over silica gel. Elution with chloroform produced 0.81g (1.5mmol) of the chloroacetamido dye. As with the other dyes, treatment with sodium iodide in boiling methanol gave the iodoacetamide, MC4.1-IA, XVI, m.p. 76–78 °C. IR (KBr) 999, 1,100, 1,169, 1,185, 1,312, 1,358, 1,408, 1,458, 1,474, 1,643, and 1,702 (amide I band) cm^{-1}.



Ia IIa

FIG. 2. Structures of the cyanine (Ia) and merocyanine (IIa) dyes synthesized in this study. The various substituents of individual dyes are given in Table 1.

RESULTS AND DISCUSSION

Nomenclature and Structure of New Dyes

A wide variety of cyanine and merocyanine dyes can be synthesized by incorporating various substituents in structures Ia and IIa at positions X, Y, and R_1 to R_3 (Fig. 2). To minimize the possible confusion we will name the dyes using the format, XX#.***-ZZ. In these names XX represents the type of polymethine dye (14): CY, for cyanines; MC, for merocyanines; OX, for oxonols; ST, for styryls; PY, for pyryliums and so on. The # term gives the number of carbon atoms in the polymethine bridge (e.g., # is 3 when m=1 and # is 5 when m=2). the ZZ term refers to the active functional group attached to the dye moiety. In this paper ZZ is IA for iodoacetamide and $IACH_2$ for iodoacetamidomethyl, but other reactive groups will also be incorporated into the dye structures, including maleimide (MAL), isothiocyanate (ITC), N-hydroxysuccinimide ester (OSU), dichlorotriazinylamine (DCT), and azide (AZ) to list but a few. When the dyes are covalently attached to a protein, the ZZ term can define the conjugate. The *** term will be a number unique for each combination of substituents in the polymethine structure, X, Y, and R_1 to R_3. This naming system for the reactive polymethine dyes will simplify referencing the many combinations of substituents possible.

Two types of polymethine dyes were synthesized in this study. A haloacetamido group was incorporated into the cyanines, Ia (Fig. 2), and merocyanines, IIa (Fig. 2), at positions R_1 or R_3. To enhance the water solubility of the cyanines, R_2 was an alkyl sulfonic acid. In the merocyanines, R_2 was a short chain alkyl group which resulted in only limited solubility in aqueous solvents for these dyes.

Synthesis of Dyes

The synthetic schemes for representative dyes are shown in Figure 3. Standard procedures were used for the synthesis of unsymmetrical cyanine and merocyanine dyes (14). The main reactions involved formation of substituted acetanilides from quaternized heterocyclic intermediates and dialdehyde anils. These ani-

Table 1
Structural and Spectroscopic Properties of New Reactive Dyes

Dye no.	Structures[a] M	X	Y	R_1	R_2	R_3	Properties[b] Abs.max (nm)	ϵ max ($\times 10^{-3}$)	Fluor. max.(nm)	Q.Y.
Cyanines										
CY5.1-IA	2	I	I	IAC_3H_6	C_4SO_3	H	650	147	668	0.40
CY3.2-IA	1	I	I	C_1	C_3SO_3	IA	565	116	590	0.07
CY5.3-IA	2	I	I	C_1	C_4SO_3	IA	662	197	689	0.12
CY5.4-IA	2	I	I	C_1	C_4SO_3	$IACH_2$	652	128	669	0.27
CY5.5-IA	2	I	O	C_1	C_3SO_3	$IACH_2$	611	139	637	0.42
CY5.6-IA	2	I	S	C_1	C_3SO_3	$IACH_2$	651	125	674	0.34
CY5.7-IA	2	I	S	C_1	C_3SO_3	IA	662	124	690	0.17
CY7.4-IA	3	I	I	C_1	C_3SO_3	$IACH_2$	752	195	773	—
Merocyanines										
MC4.1-IA	2	I	O	C_1	C_4	IA	582	119	607	0.08
MC4.2-IA	2	I	S	C_1	C_2	IA	606	155	628	0.05
MC6.1-IA	3	I	O	C_1	C_4	IA	683	78	712	0.14
MC6.2-IA	3	I	S	C_1	C_2	IA	705	175	728	0.08

[a]Structural variables relate to general structures Ia and IIa. I = isopropylidene, C_1 = methyl, C_2 = ethyl, C_4 = butyl, C_3SO_3 = sulfopropyl, C_4SO_3 = sulfobutyl, IA = iodoacetamide (ICH_2CONH-).
[b]All spectral measurements used 95% ethanol as solvent. Quantum yields of the dyes on labeled proteins may be significantly different.

Table 2
Spectral and Chromatographic Properties of Selected Reactive Dyes and Their Cysteine Conjugates

Reactive dye no.	Absorption maximum[a] Dye	Conj.	R (silica gel TLC)[b] Dye	Conj.	R (C_{18}-RPTLC)[c] Dye	Conj.
CY3.2-IA	565	564	.38	.05	.50	.66
CY5.6-IA	646	647	.36	.02	.56	.60
CY5.1-IA	646	646	.45	.03	.40	.51
CY7.4-IA	749	750	.40	.05	.42	.53
MC4.1-IA	582	582	.79	.19	.22	.31

[a]Solvent was 95% ethanol.
[b]Developing solvent was ethanol-acetic acid (19:1).
[c]Developing solvent was methanol-chloroform (2:1).

lides were then condensed with a chloroacetamide containing indoline to form the dye chromophore. The dyes were converted to the more reactive iodo derivatives using methanolic sodium iodide.

Three methods were used to link the haloacetyl group to the indoline nucleus. Quaternization of 2,3,3-trimethyl-(3H)-indole with a protected alkyl amine was the most straight forward approach (CY5.1-IA) method). Electrophilic substitution of the indoline using procedures developed by Gale and coworkers placed the acetamido group directly on the aromatic nucleus (12) or formed an acetamido-methyl substituent in which the amide group is not conjugated with the aromatic system (13).

A variety of different anilides were used in these dye syntheses. The basic heterocycles 2-methyl-benzoxazole, 2-methyl-benzothiazole, and 2,3,3-trimethyl-(3H)-indole quaternized with either 1,3-propanesultone or 1,4 -butanesultone formed cyanine dyes. 1,3-Dialkyl-barbituric acid and 1,3 -dialkyl-2-thio-barbituric acid formed merocyanines. Dyes with the heterocyclic nuclei connected by vinylene chains of varying lengths were made using the vinylogous series N,N′-diphenyl formamidine, malonaldehyde dianilide, and glutaconaldehyde dianilide. The specific structures and the spectral properties of the new polymethine dyes are given in Figure 2 and Table 1. The polymethine syntheses are versatile. Many other combinations of heterocyclic nuclei and dianils can be used to form dyes of these types.

Properties of Dyes

Cyanine and merocyanine polymethine dyes are characterised by sharp, intense absorption bands (ϵ > 100,000) and corresponding sharp emission profiles with Stokes shifts ranging from 15 nm to 25 nm. While the quantum yield of fluorescence for these dyes is typically between 0.2 and 0.4 in ethanol, we have found that their fluorescence decreases by a factor of about three in water. The absorption properties of these dyes are primarily determined by three structural features, m, Y, and R_3 in structures Ia and IIa. When the polymethine chain is lengthened by one vinylene unit (m to m+1), the absorption maximum is shifted to longer wavelength by about 100 nm. This relationship allows the synthesis of probes with very similar structural features that are excited in different regions of the spectrum. Thus, CY3.2-IA (m = 1) and CY5.3-IA (m = 2) have similar solubilities and reactivites, but have absorption maxima at 565 nm and 662 nm, respectively. Other examples of this characteristic can be found in Table 1 for both the cyanine and merocyanine dyes.

Substituents on the aromatic portion of the cyanines, R_3 in structure Ia, also affect the spectral properties of the dyes. For the substituents investigated here, alkyl substituents (R_3 = $IACH_2$) shift the spectra only slightly compared to the unsubstituted nucleus (R_3 = H). (For example, compare CY5.4-IA to CY5.1-IA.) However, conjugation of the amide with the chromophore (R_3 = IA) causes red shifts of about 10 nm in the absorption maxima and 15 nm to 25 nm in the emission spectra. (Compare CY5.7-IA (R_3 = IA) with CY5.6-IA (R_3 = $IACH_2$). The site and manner of iodoacetamide group attachment to the cyanines greatly affects the efficiency of fluorescence of these dyes. Substitutions on the chromophore ring systems decrease the observed quantum yields by as much as 70% when the amide is linked directly with the chromophore.

The synthetic flexibility of the cyanine dyes is very useful when designing new fluorophores. The excitation and emission peaks of the dyes can be selected to occur in specific regions of the spectrum. This property is important in laser illumination based systems, in experiments involving several fluorophores used together and in energy transfer experiments. Other characteristics of the cyanine dye intermediates can be used in the design of probes for specific applications. Dyes with enhanced or decreased chemical or photo-stability can be made. Symmetrical cyanine dyes containing two reactive groups can also be synthesized. The capacity of these bifunctional dyes to act as crosslinking reagents could be used to advantage. In rotational mobility studies it is sometimes desirable for the rotation of the chromophore to represent the rotational mobility of the biological macromolecule to which it is attached. Linking both ends of the fluorescent probe to the macromolecule would limit the independent movement of the label.

Reactions of Dyes

The reactivity of these iodoacetamido dyes was verified by the facile formation of dye-cysteine conjugates. The coupling reactions were complete in less than 20 min at pH 8 and gave single products with the dyes tested. Under these conditions iodoacetyl derivatives have high selectivity for sulfhydryl groups (18), which was indicated by the formation of only one product from each reactive dye. Absorption and chromatographic properties of selected dyes and their cysteine adducts are listed in Table 2. The visible spectral properties of the dyes were not altered by conversion to the sulfide derivatives (not all data shown). Due to the very strong absorption bands of the cyanine chromophore in the infrared region no differences between the reactive dyes and their cysteine conjugates were detectable. In fact, the cyanine absorption bands are so intense that the "amide 1" carbonyl stretching vibration of CY5.1-IA appears as a weak transition (1,654 cm^{-1}) next to a major cyanine band at 1,492 cm^{-1}. The polarity of the conjugates was significantly higher than the polarity of the iodoacetamide dyes allowing the straightforward demonstration of adduct formation by normal and reverse phase thin layer chromatography.

Sulfhydryl reactive cyanines have been recently used to modify -SH groups on proteins in situ. CY5.1-IA and CY5.7-IA, added as alcohol solutions, reacted with preparations of isolated rabbit skeletal muscle sarcoplasmic reticulum vesicles to induce a rapid Ca^{2+} release (23). Addition of low concentrations of the dyes (5–50 μM) was sufficient to trigger the calcium release. The labeling of particular proteins in the vesicle preparations was indicated by the occurrence of specific fluorescent bands separated by SDS polyacrylamide gel electrophoresis.

To further demonstrate the potential usefulness of these new reagents we have labeled a soluble protein, actin, with an iodoacetamido- cyanine dye. Actin is a major component of the cytoskeleton of most cells and functions by reversibly polymerizing to form filaments. Wang and Taylor have reported a method for labeling F-actin (filamentous form) specifically at Cys-374 with iodoacetamidofluorescein (29). This basic technique has been used with CY5.4-IA. In this case the dye was first adsorbed onto celite as an inert support since these dyes have limited water solubility. The recovered actin had a dye/protein ratio of 0.6, a fluorescence quantum yield of 0.13 and a maximum absorbance coefficient at 652 nm of 140,000. SDS-polyacrylamide gel electrophoresis of the product showed a single blue-colored band before staining which corresponded to the single protein band on the gel. The F- and G- states of the labeled actin had very similar excitation and emission spectra. The spectra of the labeled G-actin are shown in Figure 4. Spectra of free CY5.4-IA in the same buffer are included for comparison.

The cyanine-labeled actin had biochemical properties similar to those of the native protein. The actin analog formed filaments in vitro with a critical concentration for polymerization of 35 μg/ml. When injected into swiss 3T3 cells, the cyanine-labeled actin appeared to become associated with intracellular stress fibers in a manner similar to the incorporation of fluorescein labeled actin into stress fibers (1). Further characterization and biological applications of cyanine-labeled actin will be described elsewhere.

Cyanine and merocyanine polymethine dyes have been shown to be effective reagents for covalently labeling proteins. These dyes have high extinction coefficients, modest fluorescence yields, and moderate photostabilities. Their syntheses can be easily manipulated to produce dyes with absorption bands ranging from 500 nm to beyond 750 nm. This selection of fluorophores opens additional windows for multi-parameter fluorescence experiments. While the present iodoacetamido dyes are useful for the specific labeling of sulfhydryl groups of proteins, they are not ideal reagents for the general labeling of proteins such as antibodies. They have only moderate water solubility and most proteins have limited sulfhydryl content. Cyanine dyes with increased solubility in aqueous media and capable of reacting with protein amino groups are discussed in the following article.

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LITERATURE CITED

1. Amato P, Taylor DL: Probing the mechanism of incorporation of fluorescently labeled actin into stress fibers. J Cell Biol 102:1074–1084, 1986.
2. Aubin J: Autofluorescence of viable cultured mammalian cells. J Histchem Cytochem 27:367–43, 1979.
3. Barak LS, Webb WW: Fluorescent low density lipoprotein for observation of dynamics of individual receptor complexes on cultured human fibroblasts. J Cell Biol 90:595–604, 1981.
4. Brooker LSG, Keyes GH, Sprague RH, VanDyke RH, VanLare E, VanZandt G, White FL: Studies in the cyanine dye series XI. The merocyanines. J Am Chem Soc 73:5326–5332, 1951.
5. Brooker LSG, Keyes GH, Sprague RH, VanDyke RH, VanLare E, VanZandt G, White FL, Cressman HWJ, Dent SG: Color and constitution. X. Absorption of the merocyanines. J Am Chem Soc 73:5332–5350, 1951.
6. Brooker LGS, Sklar AL, Cressman HWJ, Keyes GH, Smith LA, Sprague RH, VanLare E, VanZandt G, White FL, Williams WW: Color and constitution. VII. Interpretation of absorptions of dyes containing heterocyclic nuclei of different basicities. J Am Chem Soc 67:1875–1889, 1945.
7. Brooker LGS, Sprague RH, Cressman HWJ: Color and constitution. VIII. Absorption of unsymmetrical carbocyanines. J Am Chem Soc 67:1889–1893, 1945.
8. DeBiasio R, Bright G, Ernst LA, Taylor DL, Waggoner AS: Five parameter fluorescence imaging: Wound healing of living Swiss 3T3 cells. J Cell Biol 105:1613–1622, 1987.
9. Drexhage KH: National Bureau of Standards Special Publication 466, Paper 80A3-894, Washington, DC, 1977, pp 33–40.
10. Ficken GE: Cyanine dyes. In: The Chemistry of Synthetic Dyes, Venkataraman K (ed). Academic Press, New York, 1971, pp 212–334.
11. Fisher NI, Hamer FM: Tricarbocyanines. J Chem Soc 189–193, 1933.
12. Gale DJ, Wilshire JFK: Fibre-reactive basic dyes. I-Polymethine dyes containing the N-chloroacetyl group. J Soc Dyers & Colorists 90:97–100, 1974.
13. Gale DJ, Lin J, Wilshire JFK: The amidomethylation and bromination of Fischer's base. The preparation of some new polymethine dyes. Aust J Chem 30:689–694, 1977.
14. Hamer FM: The Cyanine Dyes and Related Compounds. Wiley, New York, 1964.
15. Haugland RP: Covalent fluorescent probes. In: Excited States of Biopolymers, Steiner RF (ed). Plenum Press, New York, 1983, pp 29–58.
16. Jelinek CF, Kleinschmidt RF: Preparation of malonaldehyde dianils. US Patent 2549097. Chem Abstr 45:8035a.
17. Leslie R, Saxton W, Mitchison TJ, Neighbors B, Salmon E, McIntosh JR: Assembly properties of fluorescein-labeled tubulin in vitro before and after fluorescence bleaching. J Cell Biol 99:2146–2156, 1984.
18. Means GE, Feeney RE: Chemical Modification of Proteins. Holden-Day, San Francisco, 1971.
19. Murphy RF, Roederer M: Flow cytometric analysis of endocytic pathways. In: Applications of Fluorescence in the Biomedical Sciences, Taylor DL, Waggoner AS, Murphy RF, Lanni F, Birge RR (eds). Alan R. Liss, Inc., New York, 1986, pp 545–566.
20. Packard BS, Wolf DE: Fluorescence lifetimes of carbocyanine lipid analogues in phospholipid bilayers. Biochemistry 24:5176–5181, 1985.
21. Pagano RE, Sleight RG: Defining lipid transport pathways in animal cells. Science 229:1051–1057, 1985.
22. Roth NJL, Craig AC: Predicted observable fluorescent lifetimes of several cyanines. J Phys Chem 78:1154–1155, 1974.
23. Salama G, Waggoner AS, Abramson J: Sulfhydryl reagent dyes trigger the rapid release of calcium from sarcoplasmic reticulum vesicles. Biophys 47:456a, 1985.
24. Sims PJ, Waggoner AS, Wang C-H, Hoffman JF: Studies on the mechanism by which cyanine dyes measure membrane potential in red blood cells and phosphatidylcholine vesicles. Biochemistry 13:3315–3330, 1974.
25. Taylor DL, Amato PA, Luby-Phelps K, McNeil P: Fluorescent analog cytochemistry. Trends Biochem Sci 9:88–91, 1984.
26. Taylor DL, Wang Y-L: Molecular cytochemistry: Incorporation of fluorescently labeled actin into living cells. Proc Nat Acad Sci USA 75:857–861, 1978.
27. Waggoner AS: Dye indicators of membrane potential. Ann Rev Biophys Bioeng 8:47–68, 1979.
28. Waggoner AS: Fluorescent probes for analysis of cell structure, function and health by flow and imaging cytometry. In: Applications of Fluorescence in the Biomedical Sciences, Taylor DL, Waggoner AS, Murphy RF, Lanni F, Birge RR (eds). Alan R. Liss, New York, 1986, pp 3–28.
29. Wang Y-L, Taylor DL: Preparation and characterization of a new molecular cytochemical probe: 5-iodoacetamidofluorescein-labeled actin. J Histochem and Cytochem 28:1198–1206, 1980.
30. Zavortink M, Welsh M, McIntosh J: The distribution of calmodulin in living mitotic cells. Exp Cell Res 149:375–385, 1983.